Proceedings of the 27th West Indies Agricultural Economics Conference

In collaboration with

The Ministry of Agriculture and Fisheries, Belize,

Caribbean Agricultural Research and Development Institute (CARDI),

The University of the West Indies (UWI)

and

la Asociación de Latinoamérica y del Caribe de Economía Agrícola (ALACEA)

Improving Marketing and Sustaining Natural Resource Systems in Latin America and the Caribbean

Belize City, Belize

23rd - 27th July, 2007

Sharon D. Hutchinson
Editor

Antibacterial Efficacy of Eryngium foetidum (Culantro) against Select Food-borne Pathogens

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Abstract

There is a growing trend of consumer preference for the use of natural food preservatives either to prevent the growth of food-borne pathogens, or to delay the onset of food spoilage. In this investigation, an in-vitro screening method was used to determine the antibacterial efficacy of a 10% w/v suspension of the natural leaves of the herb culantro (Eryngium foetidum), against Staphylococcus aureus, Bacillus subtilis, Escherichia coli and Salmonella Typhimurium. The apparent sensitivity of the gram-positive bacteria and resistance of the gram-negative bacteria were distinct. Significant antibacterial activity was evident against S. aureus and B. subtilis, but no apparent antibacterial activity was evident against E. coli and S. Typhimurium. There was approximately 100% kill for both S. aureus and B. subtilis. The results obtained from this investigation suggest that culantro leaves can potentially be used as a food preservative, by increasing the safety and extending the shelf life of food products.

Keywords: natural food preservatives; food-borne pathogens; antibacterial efficacy; culantro leaves; in-vitro screening; % kill.

INTRODUCTION

Internationally, approximately 1400 herbs, spices and plants, have been reported to be potential sources of antimicrobials. Among the compounds having a wide spectrum of antimicrobial effectiveness are the essential oil thymol, from thyme and oregano; eugenol from cloves;

cinnamic aldehyde from cinnamon and allicin from garlic (Nychas 1996). Although substantial literature exist with respect to the antimicrobial properties of its relative coriander, there is very little information on the antimicrobial properties of the herb culantro, *Eryngium foetidum*, Family: Umbelliferae (or Apiaceae- parsley family); Common Names: long coriander leaf, thorny coriander, culantro, recao, saw leaf, chandon beni [French], shado beni [English-speaking Caribbean] and bhandhania [Trinidad & Tobago] (Ramcharan 1999).

In the Caribbean Region, the harvested natural leaves of culantro are widely used as a food flavoring and seasoning for vegetable and meat dishes, chutneys, preserves, sauces and snacks. Its medicinal value includes its use as a tea for flu, diabetes, constipation and fevers (Ramcharan 1999). Culantro is strongly flavored and pungent, and it has been established by some researchers that the more aromatic herbs exhibit greater antimicrobial properties (Hirasa and Takemasa 1998).

Objectives of study

Increased consumer preference is occurring for minimally processed and natural food products that are shelf-stable and safe for human consumption. The excessive use of chemical preservatives, many of which are suspect because of their potential carcinogenic and teratogenic attributes or residual toxicity, substantiates the use of natural preservatives like culantro, as a desirable alternative in food applications (Nychas and Tassou 1999). There is considerable interest in the possible use of such natural alternatives as food additives, either to prevent the growth of food-borne pathogens, or to delay the onset of food spoilage (Nychas 1995).

There is need for more information on the antimicrobial efficacy of the leaves of herbs. It has been documented that Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis,* are more sensitive to spices and herbs than Gram-negative bacteria such as *Escherichia coli* and *Salmonella* Typhimurium (Hirasa and Takemasa 1998). Therefore, the objective of this research was to determine the antibacterial efficacy of the freshly harvested leaves of the indigenous herb, *Eryngium foetidum*, against the common food-borne pathogens Gram positive *Staphylococcus aureus and Bacillus subtilis* and Gram negative *Escherichia coli* and *Salmonella* Typhimurium.

MATERIALS AND METHODS

Acquisition & storage of culantro leaves

Whole leaves of the culantro plant were obtained from a herbal garden located in the Sangre Grande region (Trinidad). The leaves were kept refrigerated (2 – 8ºC) in snap-sealed,

transparent, low-density polyethylene bags and used within 72h after harvesting.

Preparation of culantro leaves

As shown if Figure 1, the culantro leaves were washed under cold, running tap water to remove any extraneous matter from the surface of the leaves. The leaves were then immersed in a beaker of boiling water (100 $\pm$ 1ºC) and blanched for 2 minutes to reduce the microbial load. After blanching, the leaves were removed with sterile forceps, and placed in a single layer on a sterile tray in a laminar flow cabinet to air dry, thereby maintaining sterile conditions.

The leaves were visually examined for the absence of moisture. Using aseptic techniques, and within the confines of the laminar flow cabinet, the leaves were firstly cut into bits with sterile, stainless steel scissors, to allow ease of grinding. A steam sterilized mortar and pestle were used to manually grind the bits of leaves, to release their antimicrobial active components.

Test micro organisms

The four (4) commercially obtained (Oxoid, Hampshire, England) food-borne pathogenic bacteria used in the study were: (1) *Staphylococcus aureus*, ATCC (American Type Culture Collection) 6538 (derived), Oxoid Quanti-Cult Plus brand; (2) *Bacillus subtilis*, ATCC 6633 (derived), Oxoid Quanti-Cult Plus brand; (3) *Escherichia coli*, ATCC 35218 (derived), Oxoid Culti-Loop brand and (4) *Salmonella* Typhimurium, ATCC 14028, Oxoid Culti-Loop brand.

Preparation of bacterial cultures

The bacterial cultures were rehydrated according to the manufacturer's instructions (Bridson 1998). Duplicate tryptone soya broth tubes (10ml), [TSB - Oxoid, England] were inoculated with 0.1 ml of each culture suspension, to provide the working broth cultures to be used in the test method. Duplicate tryptone soya agar plates [TSA - Oxoid, England] were streaked with a loopful of each culture suspension, to verify purity of the test micro organisms by visual examination of colonies for uniformity in morphology. All tubes and plates were incubated at 35ºC for 24h and 48h, respectively. Subcultures were prepared by streaking a loopful of broth culture onto TSA test tube slants and incubating at 35ºC for 48h. Both subcultures and working cultures were refrigerated at 2-8ºC.

Quantitative suspension test

Immediately after grinding, a 10% w/v suspension was prepared according to Bagamboula et al. (2003). Aliquots (1g) of ground leaves were aseptically weighed into sterile TSB (9 ml) [sterilized by autoclaving

at 121ºC for 15 minutes], contained in wide-mouth universal bottles. Duplicate bottles were prepared for each of the 4 test micro organisms under investigation, the negative control and the unblanched leaves. All bottles were vigorously hand-shaken to obtain an even suspension of herb.

In a biological safety cabinet, each bottle was inoculated with the relevant culture suspension (0.1 ml). No inoculum was added to the TSB bottles containing the unblanched minced leaves, and the negative control, with the latter constituting only blanched ground leaves. Positive control bottles for comparative analysis to evaluate efficacy of the test samples, were also prepared by inoculating TSB (10 ml), with the culture suspension (0.1 ml), for each test bacterium. A sterility control consisting of TSB only, to verify that the medium was free from contamination, was included. All bottles were mixed and incubated at 24 $\pm$ 1ºC for 18-24 h, which was considered adequate conditions for the experiential growth of these bacterial broth cultures.

At the end of the contact time, the bottles were removed from the incubator and the contents were thoroughly hand-mixed. Using an Eppendorf pipetter and sterile tips, serial dilutions were prepared, with 1 ml of the TSB reaction mixture from each bottle into 9 ml-sterile Buffered Peptone Water (autoclaved at 121ºC for 15 minutes) diluent tubes [BPW – Oxoid, England]. The contents of the tubes were mixed and 0.1 ml volumes of the relevant dilutions were spread-plated onto pre-poured, solidified, sterile TSA (autoclaved at 121ºC for 15 minutes) plates [disposable petri dishes, 90 x 15 mm]. Surface plating technique was used since the colonial morphology of surface colonies is easily observed to distinguish any contaminating micro organisms from typical colonies (Downes and Ito 2001). Sterility control plates of TSB, BPW and TSA were included. The plates were inverted and incubated at 35 $\pm$ 1ºC for 48 $\pm$ 2 h.

At the end of the incubation period, viable CFU/ ml were enumerated. The entire procedure, except for the unblanched leaves, was replicated 3 times for all 4 test bacteria, with all analyses conducted in duplicate, in each replication.

Confirmatory tests

Gram staining was performed on isolated colonies from each cultured plate of the 4 test micro organisms. The procedure was used to differentiate intact, morphologically similar bacteria based on cell colour, cell form, size and structural details (Becton, Dickinson and Company, 2001). The slide preparations were examined microscopically under an oil immersion lens, and compared with colonies from the positive control plates.

Staphylococcus aureus.

The coagulase test was conducted on the presumptive *S. aureus* colonies from both the test plates and the

corresponding +ve control plates. Colonies were picked from the TSA plate and inoculated into 0.5ml coagulase plasma with EDTA [BD BBL, Maryland, U.S.A.] tubes, incubated at 35 ± 1°C, and observed over a six (6) hour period for coagulation. A positive control using the reference control *S. aureus*, a negative control using the reference control *B. subtilis* and a sterility control without culture to verify that the medium was free from contamination, were included (Downes and Ito 2001).

Escherichia coli.

Colonies were picked from the TSA plate and inoculated into EC broth (10ml), [*E. coli* Broth - Difco, Maryland, U.S.A.] tubes containing an inverted Durham vial. Tubes were incubated in a circulating water bath at 45 ± 0.5°C for 48 ± 2 h and observed for gas production. A loopful of culture suspension was aseptically taken from each gassing tube and streaked unto Levine Eosin Methylene Blue Agar [L-EMB, Oxoid, England] plates. Plates were incubated at 35 ± 1°C for 18 to 24 h and then examined for typical colonies. A positive control using the reference control *E. coli,* a negative control using the reference control *S. typhimurium* and a sterility control were included (USFDA 1998).

Salmonella Typhimurium

Colonies were picked from the TSA plate and inoculated by streaking and stabbing TSI [Triple Sugar Iron – Oxoid, England] agar slants, and stabbing and streaking LIA [Lysine Iron Agar – Oxoid, England] slants. Slants were incubated at 35 ± 1ºC for 24 ± 2 h. The agar slants were examined for changes in colour, indicative of acid/ alkaline production, and also for hydrogen sulphide production. A positive control using the reference control *S.* Typhimurium, a negative control using the reference control *E. coli* and a sterility control were included (USFDA 1998). TSI cultured slants were sent to a reference laboratory (Trinidad Public Health Laboratory) for serological confirmation of *S.* Typhimurium.

IMViC tests

Further biochemical tests were simultaneously conducted on presumptive *E. coli* and *S.* Typhimurium colonies. Colonies were picked from the respective TSA plates and IMViC (Indole, Methyl red, Voges-Proskauer and Citrate) biochemical tests were conducted. Each bacterium functioned as the negative control for the other. An un-inoculated medium control was used for each test to eliminate any false positives.

For the Indole test, tubes of tryptone water (5 ml) [Oxoid, England] were inoculated with isolated colonies and incubated 24 ± 2 hr at 35 ± 1.0ºC. A volume of 0.2 – 0.3 ml Kovac's reagent (USFDA – BAM, 1998) was added to each tube and observed for indole production, evident by the appearance of a distinct red color on the surface of the medium (USFDA 1998).

For Voges-Proskauer-reactive compounds, tubes of MR-VP medium (6 ml) [Oxoid, England] were inoculated with isolated colonies and incubated for 48 ± 2 hr at 35 ± 1.0ºC. After incubation, 0.6 ml of 5% alpha-naphthol solution [BDH, England] and 0.2 ml 40% KOH solution (potassium hydroxide – commercially available) were added to a 1-ml culture suspension and vigorously hand-shaken. Few crystals of creatine [Sigma, USA] were added, contents were again shaken, and the tubes kept at ambient temperature in BSC for 2 hr. The contents were observed for the development of an eosin pink colour throughout the medium (USFDA 1998).

For Methyl Red-reactive compounds, the remaining contents of the MR-VP tube were re-incubated for an additional 48 ± 2 hr at 35 ± 1.0ºC. After incubation, 5 drops of methyl red solution (methyl red in ethanol solution) were added to each tube and observed for a diffused red colour in the medium (USFDA 1998).

For utilization of Citrate, Simmon's Citrate Agar [Oxoid, England], slants were inoculated by streaking and stabbing with an inoculating needle. The slants were incubated for 48 - 96 hr at 35 ± 1.0ºC and then observed for a colour change from green to blue (USFDA 1998).

Statistics

The mean CFU/ml was calculated using data from all three (3) replicates, according to the procedure for computing and reporting by Downes and Ito (2001).

RESULTS AND DISCUSSION

The mean number of CFU/ g in the blanched (100 $\pm$ 1ºC for 2 minutes) leaves, the negative control, was below the detection limit (<10 CFU/ml), indicating that the blanching process effectively reduced contaminating micro organisms on the leaves. The absence of any viable CFU from both the sterility controls and the negative control also indicated that aseptic techniques employed throughout the procedure were effective.

The mean population count for the *S. aureus* positive control was approximately 10^9 CFU/ ml. The mean population count for the *B. subtilis* positive control was approximately 10^7 CFU/ ml. The mean population counts for both the *E. coli* and *S.* Typhimurium positive controls were approximately 10^9 CFU/ ml.

Antibacterial activity of 10 % (w/v) culantro (*Eryngium foetidum*) leaves on *S. aureus* and *B. subtilis*

As shown in Table 1, there was a 99.99% kill of *S aureus* in the test sample, when compared with the number of viable organisms in the control. The reduction is similar to the findings of Nychas (1995), in which almost all essential oils from herbs

tested against *S. aureu*s, inhibited its growth.

Reduction in the number of viable organisms was even greater for *B. subtilis*, with approximately 100% kill in the test sample, which was less than 1.0 * 10^2 CFU/ ml for_data analysis. Counts of *B subtilis* obtained for the positive control were lower than the counts obtained for the other 3 test organisms. This could be due to the optimal growth requirements of *B. subtilis*, which grows better under agitation, and in a growth medium that is richer than TSB. TSB provides only the minimal nutrients required for growth (Dahl 2000). Additionally, *B. subtilis* is a sporing bacterium and according to Dahl (2000), the sporulation process is initiated at the end of the exponential growth phase. External (and presumably also internal) signals, force the cell to respond by inhibiting cell division and initiating the sporulation process (Dahl 2000).

In a study comparing the susceptibility of microorganisms to the volatile oils of herbs, *B. subtilis* was the most susceptible to the volatile oils of dill weed and thyme. Hexane extracts of rosemary and sage were found to possess especially strong inhibitory activity against *S. aureus* and *B. cereus (*Hirasa and Takemasa 1998).

Antibacterial activity of 10 % (w/v) culantro (*Eryngium foetidum*) leaves on *E. coli* and *S.* Typhimurium

As seen in Table 2, in contrast to the significant antibacterial efficacy obtained against *S. aureus* and *B. subtilis*, there was no reduction in the counts of *E. coli* and *S.* Typhimurium. Similarly, it was reported that *E. coli* was relatively resistant to the volatile oils of dill weed and thyme (Hirasa and Takemasa 1998). According to Nevas et al. (2004), *S.* Typhimurium and *E. coli* were among the gram-negative bacteria that were most resistant to the 13 essential oils studied.

The CFU/ ml obtained was slightly greater for both *E. coli* and *S.* Typhimurium test samples (3.7 x 10^9 and 3.6 x 10^9 CFU/ ml respectively), compared to the controls (1.3 x 10^9 and 1.6 x 10^9 CFU/ ml respectively). Since the blanching process (negative control) and the sterility (media) controls showed no viable contaminating micro organisms, the increase in number of viable microbes could have been due to a possible growth-promoting factor present in the culantro leaves.

Confirmation of purity of bacteria

All colonies visually appeared to be of the same colony type on TSA plates, for each individual bacterium. Microscopic examination of the Gram-stained colonies obtained from both the test plates, as well as from the corresponding positive control plates, revealed similar characteristics.

Morphological and biochemical characteristics of the bacterial

colonies from the test and control TSA plates

The morphological characteristics observed for the 4 bacteria are outlined in Table 3. For the coagulase test, coagulation was evident within 6 hours, which is a reaction typical of coagulase-positive *S. aureus*. No coagulation was evident in the negative control (*B. subtilis*) and the sterility control tubes.

The production of gas in EC medium, when incubated at 45.5°C for 48h, is considered to be a positive reaction for the presence of faecal coliforms, as well as presumptive *E. coli* (Downes and Ito 2001). Both the test sample and the positive control gave positive reactions. No gas production was evident in the negative control (*S.* Typhimurium) and the sterility control tubes.

Colonies on L-EMB selective agar from the test sample were typical of the characteristic nucleated, dark-centered colonies with green sheen, as that of *the E. coli* colonies observed on the positive control plates (Downes and Ito 2001). There was no growth on the negative control and sterility control plates.

The reactions from the presumptive *S.* Typhimurium colonies, when inoculated on the TSI and LIA slants, were identical for both the test and the positive control. The reactions observed were typical of *Salmonella* spp. on TSI agar, which are an alkaline (red) slant and acid (yellow) butt with the production of H_2S [blackening of agar] (Downes and Ito 2001). The negative control (*E. coli*) produced an acid slant and butt (yellow throughout) and no H_2S. There was no change in appearance of the sterility control (un-inoculated medium). The reactions observed on the LIA were also typical of *Salmonella* spp. that is, an alkaline (purple) butt from the decarboxylation of lysine and production of H_2S (Downes and Ito 2001). The negative control produced an acid (yellow) butt and no H_2S. There was no change in appearance of the sterility control (un-inoculated medium).

IMViC reactions for *E. coli* and *S.* Typhimurium

Table 4 reveals that the colonies on the TSA test plate were confirmed as *E.coli* by the IMViC tests. IMViC tests are used to elicit specific biochemical reactions between certain bacteria and the reagent(s) used. The resulting interactions are considered to be either positive (+) or negative (-), and the combination of results is used in identifying the particular bacterium. The biochemical reactions obtained were identical to that obtained from the colonies on the positive control TSA plates. All presumptive cultures that give IMViC patterns of ++-- (biotype 1) or -+-- (biotype 2) are considered to be *E. coli* (USFDA 1998). No visible reaction occurred in the tests using the un-inoculated medium, indicating that there were no false positives. The reactions obtained from the IMViC tests are represented in Table 4.

IMViC tests conducted on colonies from the test plate and the +ve control plate produced identical reactions typical for *Salmonella* spp. that is -+-+ (USFDA 1998). No visible reaction occurred in the tests using the un-inoculated medium, indicating that there were no false positives. The reactions obtained from the IMViC tests are represented in Table 4. Further biochemical and serological tests conducted by the reference laboratory confirmed that the culture on the TSI slant was *Salmonella* Typhimurium.

Comparison with referenced literature

In all three (3) replications, significant antibacterial activity was evident against the gram-positive *S. aureus* and *B. subtilis.* No antibacterial activity was apparent against the gram-negative *E. coli* and *S.* Typhimurium. These findings are similar with those of Nevas et al. (2004), in which the gram-positive bacteria appeared to be more sensitive to the essential oils than the gram-negative bacteria. These researchers found that *S.* Typhimurium and *E. coli* were among the gram-negative bacteria that were most resistant to the essential oils studied. Most pungent herbs like culantro, have relatively strong antibacterial and/or antifungal properties. The majority of antimicrobial components of these pungent herbs are found in volatile components of their essential oils (Hirasa and Takemasa 1998).

Herbs, especially their essential oils, are more active against gram positive than gram negative bacteria (Davidson and Zivanovic 2003). The activity of essential oils of herbs against gram positive bacteria is bacteriocidal, while it is bacteriostatic against gram negative bacteria (Nychas and Tassou 2000). Rota et al. (2004) reported that the MIC of the essential oils from aromatic plants was lower for gram-positive *S. aureus,* than for gram-negative bacteria, including *S.* Typhimurium and *E. coli* O157:H7. Culture medium, temperature and inoculum size also influence antimicrobial activity of essential oils (Nychas and Tassou 2000).

CONCLUSION

The blanching process of 100 $\pm$ 1ºC for 2 minutes was effective in reducing the normal micro flora on the culantro leaves, to below the detection limit. The concentration of the leaves suspension, 10% w/v, and the mincing process, were effective for antimicrobial activity. The contact period and time used, 18-24h at 24 $\pm$ 1ºC respectively, provided adequate conditions to produce antimicrobial efficacy.

The test bacteria and the method selected for this study proved to have been very applicable since the difference in viability, and therefore efficacy, was distinctly evident between the gram-positive and the

gram-negative bacteria. The sensitivity of the gram-positive bacteria and the resistance of the gram-negative bacteria to the culantro leaves were clearly apparent.

The antibacterial efficacy of the leaves of the indigenous herb, *Eryngium foetidum*, against these select common food borne pathogens *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Salmonella* Typhimurium, was determined. At 10% w/v, the culantro leaves exhibited significant antibacterial efficacy against gram-positive *Staphylococcus aureus* and *Bacillus subtilis*, but not against gram-negative *Escherichia coli* and *Salmonella* Typhimurium.

Implications

The results obtained from this investigation indicated that culantro leaves do possess antimicrobial components, with significant antimicrobial activity. Therefore, in theory, culantro leaves can be used as a natural additive in the preservation of food, by increasing the safety and extending the shelf life of food products. Culantro's characteristic intense flavour may also enhance the organoleptic properties in food products such as meat, sauces, dressings and condiments.

Recommendations for future research

- To determine the MIC of the natural culantro leaves on these test micro organisms.
- To determine the MIC of the essential oil on these test micro organisms.
- To determine the MIC of dried culantro leaves on these test micro organisms.
- To determine the effect on efficacy by varying parameters such as contact time and temperature.
- To determine the antimicrobial efficacy on other food-borne pathogenic and spoilage bacteria, as well as fungi.
- To determine the antioxidant properties of the active components in the culantro plant.
- To determine the antimicrobial efficacy in real food products, conduct sensory evaluations and perform comparative analysis on impact on shelf life.
- To use other test methods for comparative analysis of efficacy.
- To test and compare efficacy with other locally grown herbs.

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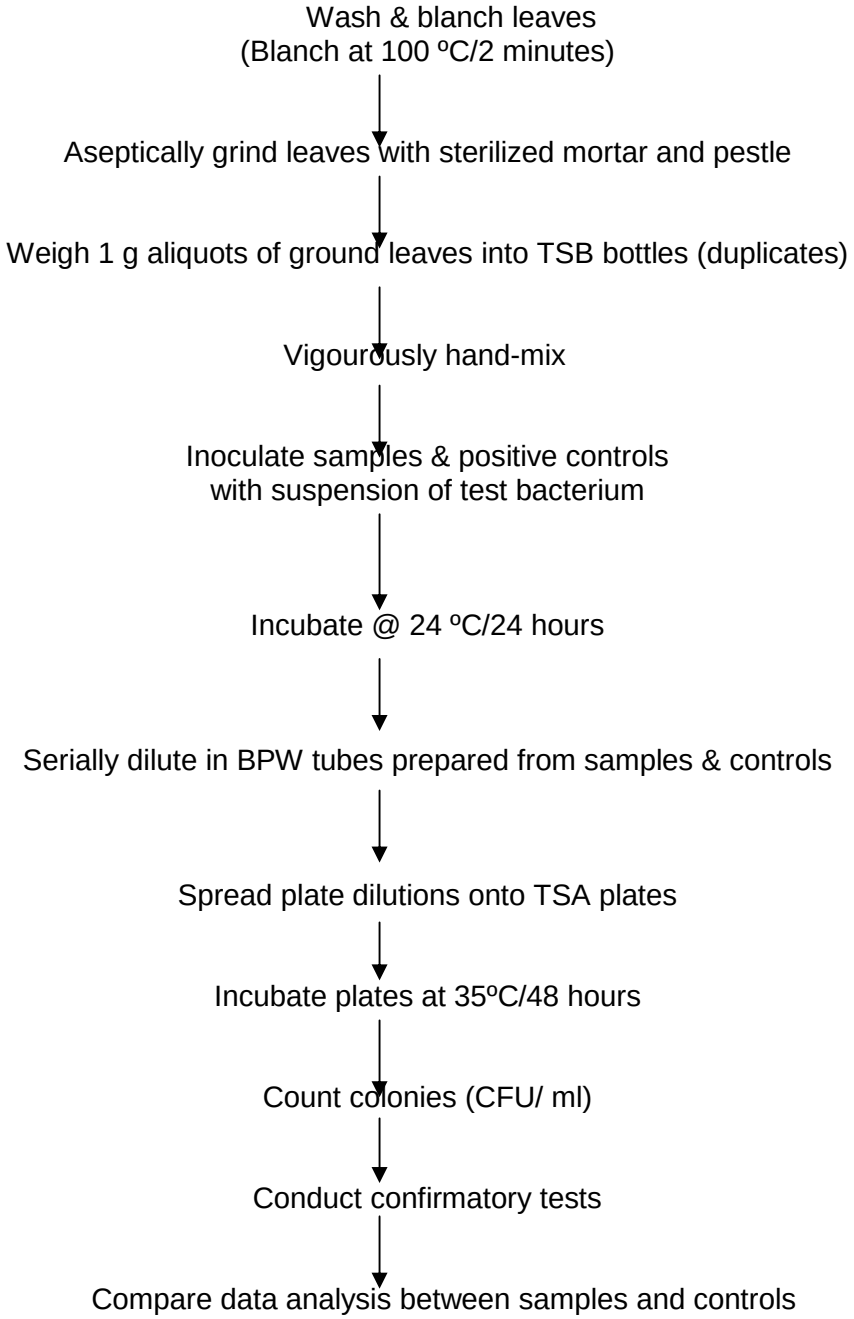

Figure1. Flow Chart for Determining Antibacterial Efficacy of *Eryngium foetidum* (Culantro)

Table 1: Antibacterial Activity of 10 % (w/v) Culantro (*Eryngium foetidum*) Leaves on *S. aureus* and *B. subtilis.*

Gram Positive Test Bacteria	*S. aureus*		**B. SUBTILIS**	
	Test	Control	Test	Control
Mean CFU/ ml	1.2×10^5	2.0×10^9	1.0×10^2	3.9×10^7
% Kill	99.99		100	

Source: Compiled by author

Table 2: Antibacterial Activity of 10 % (w/v) Culantro (*Eryngium foetidum*) Leaves on *E. coli* and *S.* Typhimurium.

Gram Negative Test Bacteria	*E. coli*		**S.*TYPHIMURIUM***	
	Test	Control	Test	Control
Mean CFU/ ml	3.7×10^9	1.3×10^9	3.6×10^9	1.6×10^9
% Kill	0		0	

Source: Compiled by author

Table 3: Morphological Characteristics of the Bacteria Colonies from both the Test and Control TSA Plates

Test and Positive Control	TSA	Gram Stain
Staphylococcus aureus	Yellow colonies, smooth margin	Gram positive, large cocci in clusters
Bacillus subtilis	White colonies, irregular margin, dry, flat	Gram positive, sporing rods, central spores
Escherichia coli	Cream colonies, smooth margin	Gram negative, rods
Salmonella Typhimurium	Cream colonies, smooth margin	Gram negative, rods

Source: Compiled by author

Table 4: IMViC reactions for *E. coli* and *S.* Typhimurium

Test and Control	I	M	V	C
Test *E. coli*	+	+	-	-
Control *E. coli*	+	+	-	-
Test *S.* Typhimurium	-	+	-	+
Control *S.* Typhimurium	-	+	-	+
Sterility Control Un-inoculated	No change	No change	No change	No change

Source: Compiled by author